PRE-STEADY STATE KINETICS OF NUCLEOTIDE-TRIPHOSPHATE HYDROLYSIS BY MITOCHONDRIAL F_1-ATPase FROM YEAST

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1. Introduction

In [1] we described the observation of a time-delayed onset of F_1-ATPase activity of yeast (YF_1) which indicated a conformational transition of the enzyme complex by a substrate-triggered activation mechanism. This observation was later confirmed for F_1-ATPase from *Neurospora* as well as thermophilic bacteria (TF_1) [2] and by others for the case of F_1-ATPase of beef-heart [3] and chloroplast [4].

In a search for the mechanism of this conformation change we recently analyzed the sensitivity of the lag time toward nucleotide triphosphates, ADP, pH and anions, and observed only a sensitivity toward ADP. The results of these experiments are reported here.

2. Methods

Nucleotide di- and triphosphates were from Boehringer/Mannheim, ϵ-ATP from Sigma, phenol red and cresol red form Serva, Heidelberg. All other chemicals were of the purest grade commercially available. Nucleotides and their analogs were determined spectrophotometrically.

The enzyme was prepared according to [5] and stored as suspension in 70% $(NH_4)_2SO_4$, 4 mM ATP, 50 mM Tris–HCl, 2 mM EDTA. To transfer the enzyme into buffer for stopped-flow experiments the suspension was spun down at 100 000 × *g* for 90 min and the pellet dissolved in the appropriate buffer. The resulting solution was desalted through G-25 or G-50 gel equilibrated with buffer according to [6]. Protein concentrations were obtained from the activity, the specific activity and the molecular weight as given in [5].

H^+ liberation was measured in stopped-flow experiments using the indicators phenol red or cresol red. The experiments were performed using a Durrum stopped-flow instrument with a 50 W tungsten lamp and a 2 cm quartz fluorescence cuvette in transmission mode. The dead-time of the instrument was < 2 ms as determined by the ferrocyanide ascorbate reaction. The change in pH was monitored by following the absorption at 558 nm for phenol red and at 573 nm for cresol red. From the curves thus obtained the lag time of the reaction was determined.

ϵ-ATP hydrolysis was measured in the presence of Mn^{2+} [7] by monitoring the change in ϵ-ATP fluorescence in a Durrum stopped-flow instrument in fluorescence mode. The excitation was at 310 nm (prism), the light emitted at rightangle to the excitation was passed through a filter absorbing below 395 nm. The slitwidth for the excitation was 5 mm.

The amplifier output of the stopped-flow instrument was fed to a Nicolet 1074 digital storage oscilloscope connected to a Wang 2200 calculator, which was used for data storage on discette and linear and logarithmic data plotting with a drum plotter with BASIC software specially developed for that purpose (D.R., B.H., unpublished).

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3. Results

It has been shown that ATP hydrolysis by F_1-ATPase is preceded by a lag time [1–4]. In order to analyze the sensitivity of the lag time toward the ATP concentration in a wide concentration range the pH-indicator technique was used as shown in fig.1 illustrating a lag time of 70 ms followed by a pH change being linear for ~ 500 ms before slowing down due to substrate exhaustion in a first-order exponential fashion. The experiment demonstrates that ATP-hydrolysis begins after a lag time of ~ 70 ms. As shown in table 1 experiments performed at different concentrations of nucleotide triphosphates revealed that the lag time is independent of the substrate concentration, thus indicating a first-order process.

When the pH-indicator technique was supplemented by the use of ϵ-ATP as a fluorescent ATP analog with similar affinity to YF_1 [8], a lag time of 150 ms was observed before ϵ-ATP hydrolysis sets in, consistent with the results obtained by using a pH-indicator. The ϵ-ATP method proved to be useful to study the dependence of the observed lag time on the substrate and the enzyme concentration. As shown in fig.2 no significant influence of substrate at 2–200 μM on the duration of the lag time was found, confirming the conclusion of a first-order reaction course for the activation. This observation was further substantiated by the finding that the enzyme at 0.1–0.5 μM does not affect the lag time.

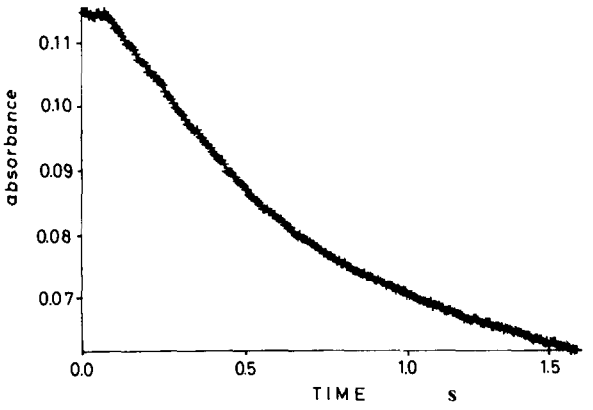

Fig.1. Stopped-flow absorbance trace at 558 nm obtained after mixing 2 μM YF_1 (30 U/ml) with 500 μM ATP, both in a solution containing 2 mM $MgCl_2$ and 10 μM phenol red at pH 8 (optical pathlength 20 mm; multiplier voltage, 446 V; monochromator slitwidth, 0.16 mm; temp., 25°C).

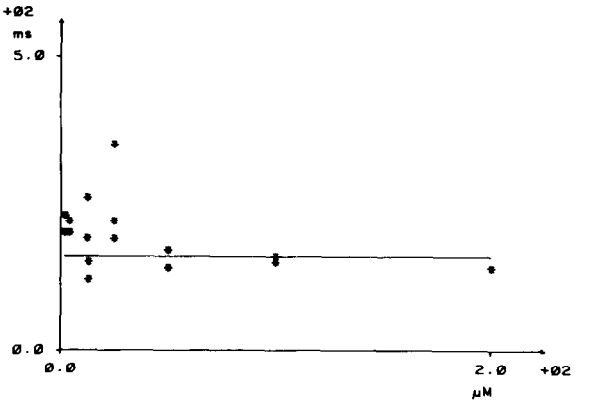

Fig.2. Dependence of the lag time of Mn-ϵ-ATP hydrolysis on the substrate concentration (50 mM HEPPS . K, 2 mM $MnCl_2$, 0.5 μM F_1).

Since, in earlier experiments, we observed two different anion-induced states in the steady state kinetics of YF_1 [9] we tested the sensitivity of the lag time towards a number of anions, however, no significant influence of HSO_3^- (t = 120 ms), HCO_3^- (t = 110 ms) and SO_4^{2-} (t = 150 ms) was observed, thus demonstrating that both conformations of YF_1 have to be activated before ATP hydrolysis can occur.

As shown in table 2 the lag time was also independent of the pH at 6.6–8.6. This is of interest because it excludes the participation of tightly bound nucleotides in process producing the lag time because of the pH sensitivity of the dissociation of tightly bound nucleotides [10]. This finding can be further substantiated by the measurement of the specificity of the lag time with respect to different nucleotide

Table 1
Lag time of Mg-ATP hydrolysis

c_{ATP}	(μM)	100	250	500	1000
t	(ms)	50	70	70	60

Table 2
Lag time of Mn-ϵ-ATP hydrolysis different pH

pH	6.6	7.6	7.8
ms	150	150	130

Table 3
Lag time of hydrolysis of different nucleotide triphosphates

Nucleotide triphosphate	ATP	d-ATP	ϵ-ATP	ITP
mM	0.25	0.25	0.10	1.0
ms	70	70	150	300

triphosphates using the pH-indicator. Table 3 shows that a lag time precedes the hydrolysis of all tested nucleotide triphosphates, thus excluding the participation of tightly bound ADP, which only can be displaced by adenine nucleotide triphosphates [8,11]. The same conclusion can also be drawn from the observation that the ATP hydrolysis by TF_1, which does not contain tightly bound nucleotides, is preceded by a lag time of 2 s at 25°C. The longer lag time for TF_1 is probably due to the fact that the measurements were carried out at 25°C much below the optimal temperature for this enzyme. Indeed with YF_1 a shortening of the lag was found, when the temperature was increased (D.R., B.H., unpublished).

Since, ADP is a competitive inhibitor of the ATPase reaction [2], and also reduces the ATPase activity in a non-competitive fashion when bound to high affinity sites [11], its influence on the lag time is of interest. We, therefore, analysed the pre-steady state kinetics of ϵ-ATP hydrolysis with YF_1 samples which were preincubated in the presence of 100 μM ADP, and found a prolonged lag time of 2 s. Under the same conditions, the rate of steady state ϵ-ATP hydrolysis was reduced to 2% as compared to the non-preincubated control sample. This experiment indicated that ADP might form an inactive complex with F_1-ATPase which is converted to an active ATPase by the action of ATP with a time constant of 0.5 s^{-1}. It might well be that this ADP · F_1 complex is of importance in ATP synthesis.

4. Conclusion

The experiments presented here clearly show that F_1-ATPases from different organisms do not have hydrolytic activity before being activated by a reaction with nucleotide triphosphates as evidenced by a lag time in the hydrolysis of nucleotide triphosphates. This lag time accounts for more than 20 single turnover times. Our finding is confirmed by independent work on F_1-ATPase of beef-heart [3,12]. The lag time described in [3,12] is explained by the time required to displace tightly bound ADP by added nucleotide triphosphate.

Three observations can be taken as evidence against such participation of tight ADP sites in the activation process:

(1) The tight sites exhibit a much higher nucleotide specificity [11] than the activation by nucleotide triphosphates as shown by the lag ATP hydrolysis;
(2) The dissociation rate of tightly bound nucleotides is pH-dependent [10] whereas the lag time is not in the pH-range of 6.6–8.0;
(3) A lag time of 2 s is observed in Mg-ATP hydrolysis by TF_1 which does not contain any tightly bound nucleotides [13].

For the explanation of these results we propose a scheme where the binding of nucleotide triphosphate is followed by a conformational change thus activating the ATPase. This activation step could also be a phosphoryl transfer from a nucleotide triphosphate bound at a site of low specificity to the ADP of a tight site. The lack of any significant influence of anions, which bind to regulatory sites of F_1 [9,14], on the lag time excludes their participation in the activation process. Therefore at present none of the nucleotide binding sites can be assigned to the activation step which manifests itself by a 50–200 ms lag time.

A much longer lag was observed in the reaction of F_1 preincubated with ADP. We attribute this lag time to the displacement of bound ADP with a time constant of 0.5 s^{-1}. This reaction is also apparent from the initially reduced ATPase activity with F_1 preincubated in the presence of ADP using conventional techniques of ATPase assay [10]. Figure 3 summarizes a reaction scheme including both activating reaction steps. Normally, the reaction sequence starts from the free enzyme (E). The conversion from ATP · E to ATP · E′ leads to the observed lag time of 100 ms,

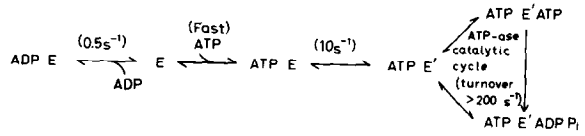

Fig.3. Reaction scheme for ATPase activation.

before ATP · E′ catalyzes ATP hydrolysis with a turnover number $> 200\ s^{-1}$. However, when the reaction starts from ADP · E, the above sequence is preceded by a lag time of 2 s while ADP dissociates from ADP · E.

The question whether the deadend path of this scheme is involved in the reverse reaction of the ATPase thus the ATP synthesis reaction, or whether it has regulatory functions, has to be solved by further experimentation, including reconstituted membrane-bound systems.

Acknowledgements

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